## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Appl. No. | : | 10/567,057 | Confirmation No. 4884 |
| Applicant | : | Gert Daube | |
| Filed | : | June 26, 2006 | |
| Title | : | Novel Use of Quinolone Antibiotics | |
| Group Art Unit | : | 1614 | |
| Examiner | : | Savitha Rao | |
| Docket No. | : | LEA 36807 | |

**VIA EFS**

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Commissioner for Patents
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Alexandria, VA 22313-1450

## DECLARATION UNDER 37 C.F.R. §1.132

Dr. Bernd Stephan, declares and states as follows:

1. I received a state exam degree in veterinary medicine in 1992 from Hanover University. Thereafter, I received a doctor's degree in veterinary medicine in 1995 from Hanover University.

2. From 1996 to present, I was employed by Bayer HealthCare AG and then subsequently Bayer Animal Health GmbH. My present position is Head of R&D Antiinfectives, with Bayer Animal Health GmbH.

3. Under my direction and control, a study to compare the minimum inhibitory concentration (MIC) of pradofloxacin and des-cyanopradofloxacin of 31 anaerobic bacterial pathogens isolated from cases of canine periodontal disease was conducted.

4. The MIC of each test article was determined against a total of 31 bacterial strains, comprising 10 strains each of Prevotella, Prophyromonas, and 11 Fusobacterium strains isolated from cases of periodontal disease in dogs. The test system was standardized agar dilution MIC methodology, as described by the Clinical and Laboratory Standards Institute (CLSI).

5. The material and methods for the study are detailed in study report attached as Attachment 1.

6. Pradofloxacin was demonstrated to be more active than des-cyano pradofloxacin against the bacterial strains tested in this study, and implicated in periodontal disease. (See Tables 1-5 of Attachment 1) Based on the geometric mean MIC, pradofloxacin activity was six-fold higher than that of the des-cyano derivative.

7. Thus it can be concluded that the high in-vitro activity of pradofloxacin against anaerobic bacteria is attributable to the cyano group at the C-8 position of this veterinary fluoroquinolone.

8. The applicant further declares that all statements made herein are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Dr. Bernd Stephan

07/05/2010
Date

don whitley scientific
Don Whitley Scientific Limited, 14 Otley Road, Shipley, West Yorkshire, BD17 7SE, UK
Tel: +44 (0)1274 595728 Fax: +44 (0)1274 531197 www.dwscientific.co.uk

DWS Study No: 038/08
BAH reference: 146.057


Report No: DWS/038/08

ID 32864

**Study Title: Determination of the Minimum Inhibitory Concentration (MIC) of pradofloxacin and des-cyano-pradofloxacin against anaerobic bacteria isolated from cases of canine periodontal disease**

Author: Andrew Pridmore BSc PhD (Study Director)
Don Whitley Scientific Limited

Report authorized by Study Director:

A Pid ............................................ 5 DECEMBER 2008

Dr Andrew Pridmore
Don Whitley Scientific Limited

Date

| DISTRIBUTION | |
|---|---|
| 1. | Bayer Animal Health GmbH |
| 2. | Archive (DWS) |

## SUMMARY

The Minimum Inhibitory Concentration (MIC) of two pradofloxacin derivatives (pradofloxacin and des-cyano-pradofloxacin) were determined against 31 anaerobic bacterial isolated from cases of canine periodontal disease. MIC determinations were performed using agar dilution MIC methodology (supplemented Brucella Blood agar) in accordance with CLSI guidelines M31-A3 and M11-A8. Activity of each test article against the strains tested in the present study is summarized below:

| Bacterial species | Summary MIC Parameter | Value (µg/ml) for each test article | |
|---|---|---|---|
| | | Pradofloxacin | Des-cyano-pradofloxacin |
| *Fusobacterium* spp. (11) | MIC Range | 0.062 to 0.5 | 0.5 to 4 |
| | $MIC_{50}$ | 0.125 | 0.5 |
| | $MIC_{90}$ | 0.5 | 4 |
| | Geometric mean | 0.17 | 1.1 |
| *Prevotella* spp. (10) | MIC Range | 0.016 to 0.125 | 0.062 to 1 |
| | $MIC_{50}$ | 0.062 | 0.5 |
| | $MIC_{90}$ | 0.125 | 0.5 |
| | Geometric mean | 0.062 | 0.38 |
| *Porphyromonas* spp. (10) | MIC Range | 0.031 to 0.062 | 0.031 to 0.5 |
| | $MIC_{50}$ | 0.062 | 0.5 |
| | $MIC_{90}$ | 0.062 | 0.5 |
| | Geometric mean | 0.054 | 0.31 |
| All strains (31) | MIC Range | 0.016 to 0.5 | 0.031 to 4 |
| | $MIC_{50}$ | 0.062 | 0.5 |
| | $MIC_{90}$ | 0.5 | 4 |
| | Geometric mean | 0.085 | 0.52 |

Pradofloxacin was demonstrated to be more active than des-cyano-pradofloxacin against anaerobic bacteria implicated in periodontal disease. Based on the geometric mean MIC, pradofloxacin activity was six-fold higher than that of the des-cyano derivative. Thus, it can be concluded that the high in-vitro activity of pradofloxacin against anaerobic bacteria is attributable to the cyano group at the C-8 position of this new veterinary fluoroquinolone.

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# 1. INTRODUCTION

## 1.1 Study Objective

To determine the Minimum Inhibitory Concentrations (MICs) of two pradofloxacin derivatives (pradofloxacin and des-cyano-pradofloxacin) against anaerobic bacterial pathogens isolated from cases of canine periodontal disease.

## 1.2 Description of Test System

The MIC of each test article was determined against a total of 31 bacterial strains, comprising 10 strains each of *Prevotella*, *Porphyromonas* and 11 *Fusobacterium* strains isolated from cases of periodontal disease in dogs during previous trials conducted by the Sponsor.

The test system was standardized agar dilution MIC methodology, as described by the Clinical and Laboratory Standards Institute (CLSI).

## 1.3 References

CLSI document M11-A7: Methods for antimicrobial susceptibility testing of anaerobic bacteria; Approved Standard - Seventh Edition (January 2007)

CLSI document M31-A3: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard - Third Edition (February 2008)

EMEA/CVMP/627/01 - Guideline for the demonstration of efficacy for veterinary medicinal products containing antimicrobial substances (Adopted by CVMP 11 December 02).

# 2. JUSTIFICATION

This is one of a series of *in vitro* studies conducted to provide efficacy data for these test articles.

## 3. RESPONSIBILITIES

### 3.1 Study Director

| | |
|---|---|
| Name: | Andrew Pridmore BSc PhD |
| Address and Telephone: | See 3.3 below |
| E-mail: | andrew_pridmore@dwscientific.co.uk |

### 3.2 Key Laboratory Personnel

| | | |
|---|---|---|
| Andrew Pridmore BSc PhD | - | Head of Microbiology |
| Andrew Shaw BSc | - | Microbiologist |
| Jacqueline Key BSc | - | Microbiologist |
| Vicki Springthorpe BSc | - | Microbiologist |
| Alison Cheetham BSc | - | Microbiologist |
| Orysia Chymera BSc AIBMS | - | Microbiologist |

The address and telephone number for these personnel is shown in 3.3 below.

Other supervised members of the DWS Microbiology Group also contributed to the laboratory phase of this study.

### 3.3 Name and Address of Facility Performing the Study

(This is also the address of the Study Director and all laboratory personnel)

Don Whitley Scientific Limited (DWS)
14 Otley Road
Shipley, West Yorkshire
BD17 7SE
United Kingdom

| | |
|---|---|
| Telephone: | +44 (0)1274 595728 |
| Fax: | +44 (0)1274 531197 |

### 3.4 Name and Address of Sponsor

Bayer Animal Health GmbH
51368 Leverkusen
Germany

### 3.5 Sponsor's Representative

Dr Hans-Robert Hehnen
Bayer Animal Health GmbH
Gebäude 6700
Alfred-Nobel-Strasse 50
D-40789 Monheim
Germany

| | |
|---|---|
| Telephone: | +49 2173 38 7727 |
| Fax: | +49 2173 38 969 7727 |
| E-mail: | hans-robert.hehnen@bayerhealthcare.com |

3.6 Quality Assurance

| | |
|---|---|
| Appointed QA Consultant: | Sally White (Optimum Quality Consultancy) |
| Telephone: | +44 (0)1423 567512 |
| E-mail: | sallywhite@optimumquality.co.uk |

3.7 GLP Compliance

This study was conducted in accordance with the OECD principles of Good Laboratory Practice. However, GLP compliance is not a mandatory requirement for this type of study, therefore GLP compliance is not claimed.

The appointed Quality Assurance consultant audited the protocol, raw data and study report. Process based laboratory audits are performed for all critical study procedures in accordance with DWS standard QA procedures.

3.8 Archiving

Following issue of the final report, the following materials will be copied and sent to the Sponsor for Archiving. The original materials will be transferred to the DWS archive at the address shown in 3.3 above:

- Originally signed final report
- Originally signed protocol, protocol amendments and notes to file
- Protocol amendments and notes to file
- All original raw data generated in relation to the study, including the designated Laboratory Notebook but not including laboratory equipment records and check sheets
- Study correspondence
- Certified copy of test substance usage records

All archive materials will be retained for 5 years after study completion.

All bacterial strains used in the study will be retained in the DWS culture collection for a minimum of 5 years after study completion. Sponsor approval will be obtained prior to disposal.

3.9 Study Duration

| | |
|---|---|
| Study initiation (protocol issue): | 8 September 2008 |
| Experimental work commenced: | 25 September 2008 |
| Experimental work completed: | 15 October 2008 |
| Study completion: | 5 December 2008 |

## 4. MATERIALS AND METHODS

Experimental work and results (raw data) were recorded in the designated DWS Laboratory Notebook M533A. Batch numbers were recorded for culture media and reagents used in each experiment during the laboratory phase of the study.

### 4.1 Protocol Amendments and deviations

No Amendments to Protocol were issued for this study.

### 4.2 Test articles

The Sponsor supplied each of the test articles used in this study. The following batches of each test article were used:

| Description | Bayer lot number | Assay for use |
|---|---|---|
| Pradofloxacin | BXR3G61 | 98.9% |
| Des-cyano-pradofloxacin | HLR7743-2-1 | 91.1% |

A Certificate of Analysis (CoA) for each test article is included in the Study Protocol (Appendix 1). Test articles were stored at room temperature, as specified by the Sponsor.

### 4.3 Bacterial strains

4.3.1 The activity of each test article was to be determined against 30 strains of anaerobic bacteria, comprising 10 strains each of *Prevotella* and *Porphyromonas* and 10 *Fusobacterium* strains (see attached protocol). Two strains were found to be non-viable on subculture from frozen stocks, therefore alternative strains were included. Finally 31 strains comprising 10 strains each of *Prevotella* and *Porphyromonas* and 11 *Fusobacterium* strains were included in this study.

4.3.2 All 31 strains were isolated from cases of canine periodontal disease during previous studies conducted by the Sponsor. Each strain was lodged in the DWS culture collection prior to initiation of the present study and is identified by its unique "DWC" strain code. All bacterial strains were routinely stored in a cryoprotective suspension at a nominal temperature of -80°C. Strains used in the study are listed below:

| Bacterial strain | DWC code |
|---|---|
| *Fusobacterium nucleatum* | DWC 5785 |
| *Fusobacterium nucleatum* | DWC 5780 |
| *Fusobacterium nucleatum* | DWC 5783 |
| *Fusobacterium nucleatum* | DWC 5792 |

| Bacterial strain | DWC code |
|---|---|
| *Fusobacterium nucleatum* | DWC 5763 |
| *Fusobacterium nucleatum* | DWC 5790 |
| *Fusobacterium nucleatum* | DWC 5736 |
| *Fusobacterium naviforme* | DWC 6957 |
| *Fusobacterium necrogenes* | DWC 5732 |
| *Fusobacterium ulcerans* | DWC 7592 |
| *Fusobacterium varium* | DWC 6924 |
| *Porphyromonas gingivalis* | DWC 11026 |
| *Porphyromonas gingivalis* | DWC 12313 |
| *Porphyromonas gingivalis* | DWC 10394 |
| *Porphyromonas gingivalis* | DWC 10791 |
| *Porphyromonas gingivalis* | DWC 11040 |
| *Porphyromonas gingivalis* | DWC 10805 |
| *Porphyromonas gingivalis* | DWC 10686 |
| *Porphyromonas gingivalis* | DWC 11834 |
| *Porphyromonas gingivalis* | DWC 11837 |
| *Porphyromonas gingivalis* | DWC 10799 |
| *Prevotella intermedia* | DWC 10891 |
| *Prevotella intermedia* | DWC 12346 |
| *Prevotella intermedia* | DWC 11487 |
| *Prevotella intermedia* | DWC 16229 |
| *Prevotella intermedia* | DWC 11027 |
| *Prevotella intermedia* | DWC 11074 |
| *Prevotella denticola* | DWC 11087 |
| *Prevotella denticola* | DWC 10397 |
| *Prevotella denticola* | DWC 10504 |
| *Prevotella denticola* | DWC 10761 |

4.3.3 Control Organisms:

The following anaerobic bacterial strains were used to monitor performance and reproducibility of the MIC test:

| | |
|---|---|
| *Bacteroides fragilis* | DWC 9674 (ATCC 25285) |
| *Bacteroides thetaiotaomicron* | DWC 14809 (ATCC 29741) |

MIC test performance was monitored on the basis of MIC results obtained against these control strains (see 5.5 below).

4.4 Culture media, media supplements and other reagents)

(Abbreviations for subsequent use are shown in the right-hand column)

| | | |
|---|---|---|
| Maximum Recovery Diluent (LabM; LAB103) | - | MRD |
| Fastidious Anaerobe Agar (LabM; LAB 90) | - | FAA |
| Sheep blood, defibrinated (Southern Group Laboratory) | | |
| Horse blood, defibrinated (Southern Group Laboratory) | | |
| *Brucella* Agar Base (Difco; D0964-17) | | |
| Hemin (Sigma-Aldrich; H-2250) | | |
| 3-phytylmenadione [Vitamin $K_1$] (Sigma-Aldrich; M-2518) | | |
| Sodium hydroxide, 1 M, 'AnalaR' Grade (BDH; 19139) | | |

Each culture medium was prepared in accordance with the manufacturer's instructions and the DWS Media Preparation Manual. After autoclaving and cooling to 47±2°C, Fastidious Anaerobe Agar was supplemented with 5% v/v defibrinated horse blood before pouring into sterile petri dishes.

4.4.1 **Preparation of supplemented Brucella Blood Agar (BBA):**

(i) Hemin stock solution was prepared by dissolving 0.5 g hemin in 10 ml of 1 M sodium hydroxide. The volume was made up to 100 ml with deionized water and the solution was autoclaved at 121°C for 15 minutes. This solution was stored at 5±3°C for not longer than 1 month.

(ii) Vitamin $K_1$ (3-phytylmenadione) solution was prepared by dissolving 0.2 g 3-phytylmenadione in 20 ml of 95% ethanol. The final solution was then prepared by adding 1 ml of the vitamin K solution to 9 ml of sterile deionized water. This solution was stored at 5±3°C for not longer than 1 month.

(iii) To prepare the complete agar, dehydrated Brucella agar base was dispersed in deionized water at the manufacturer's recommended concentration. A 1 ml volume of hemin stock solution and 1 ml of Vitamin $K_1$ (3-phytylmenadione) stock solution was added per 1 litre of agar. Agar was brought to the boil with constant stirring to dissolve the powder and be dispensed and sterilized as described in 4.6 below.

(iv) Laked sheep blood for addition to supplemented BBA was prepared by freezing defibrinated sheep blood in suitable aliquots at –20°C or below. Sheep blood was then thawed, either by placing in an incubator or water bath at approximately 37°C or by storing overnight in a refrigerator at 2°C to 8°C. After thawing, laked blood was thoroughly mixed and warmed in a 47°C water bath before adding to molten BBA as described in 4.6.3 below.

4.5 Procedure: preparation of test article stock solution

4.5.1 For each test article, the Certificate of Analysis was consulted and the target mass was corrected to account for potency / purity / assay of the

supplied material, as appropriate. A mass of the solid material equivalent to 0.2560 g (acceptable range 0.2550 g to 0.2570 g) of the active component was accurately weighed and transferred to a 50 ml volumetric flask.

4.5.2 Test articles were dissolved in deionised water. Dissolved test articles were then made up to the required volume with deionised water. Thus, final test article concentration in each solution was 5120 µg/ml (nominal).

4.5.3 Each test article stock solution was divided into appropriate aliquots (approximately 4 – 5 ml) in sterile polypropylene screw-capped tubes. Each tube was labelled with the study number, test article name, concentration and expiry date. The expiry date of these solutions was designated as 1 month from the date of preparation. All tubes were placed in frozen storage at a nominal temperature of -20°C.

Note: when required, volumes of stock solution prepared were adjusted to suit requirements to avoid unnecessary waste.

4.6 <u>Experimental conditions: general notes</u>

Inoculation and incubation of all microbiological culture media was performed within an anaerobic workstation (Don Whitley Scientific Limited) operating at 35±1°C with an atmosphere of 80% $N_2$, 10% $CO_2$ and 10% $H_2$. All culture media, including diluents, was "pre-reduced" before use by overnight storage in the anaerobic workstation. The caps of all vessels were fitted loosely, to preserve sterility while also allowing gaseous exchange with the anaerobic atmosphere. Pre-reducing simultaneously allowed pre-warming of the media prior to inoculation. All inoculated culture media was labelled with study number, organism code number, date of subculture and test article concentration (where applicable) on the bottle or petri dish.

4.7 <u>Procedure: preparation of test plates</u>

4.7.1 Dilution of test article:

On each day that MIC tests were to be performed, a single tube of each test article stock solution was removed from the freezer and the contents were allowed to thaw at room temperature. Thawed stock solution was mixed thoroughly by vortexing (any observable precipitate was dissolved) and was then used to prepare working dilutions of test article for addition to culture media. Working dilutions were prepared in sterile deionized water (SDW) using the following dilution scheme:

| Solution | Preparation method | Nominal test article concentration (µg/ml) |
|---|---|---|
| A | 2.0 ml stock solution + 6.0 ml SDW | 1280 |
| B | 1.0 ml Solution A + 7.0 ml SDW | 160 |
| C | 1.0 ml Solution B + 7.0 ml SDW | 20 |
| D | 1.0 ml Solution C + 7.0 ml SDW | 2.5 |
| E | 1.0 ml Solution D + 7.0 ml SDW | 0.31 |

4.7.2 Supplemented Brucella Blood Agar (BBA) was used for MIC testing. Agar was prepared and autoclaved at 121°C for 15 minutes in 17.0 ml aliquots.

4.7.3 After autoclaving, all agar aliquots were cooled in a water bath operating at 47±2°C. When cooled to this temperature, each 17.0 ml aliquot was aseptically supplemented with 1.0 ml of laked sheep blood, to produce a final volume of 18 ml. Blood was gently mixed with the agar by inversion (formation of bubbles was avoided).

4.7.4 Immediately after addition of blood, test article dilutions were added to individual aliquots of agar in the volumes shown below. Final concentrations of test article in the agar (by calculation) are shown in the right hand column:

| | | Test article concentration (µg/ml) | |
|---|---|---|---|
| | | Nominal (for MIC reporting) | Actual concentration |
| Solution A 1280 µg/ml | - 2.0 ml into 18 ml agar | 128 | 128.0 |
| | - 1.0 ml into 18 ml agar | 64 | 67.4 |
| | - 0.5 ml into 18 ml agar | 32 | 34.6 |
| Solution B 160 µg/ml | - 2.0 ml into 18 ml agar | 16 | 16.0 |
| | - 1.0 ml into 18 ml agar | 8 | 8.4 |
| | - 0.5 ml into 18 ml agar | 4 | 4.3 |
| Solution C 20 µg/ml | - 2.0 ml into 18 ml agar | 2 | 2.00 |
| | - 1.0 ml into 18 ml agar | 1 | 1.05 |
| | - 0.5 ml into 18 ml agar | 0.5 | 0.54 |
| Solution D 2.5 µg/ml | - 2.0 ml into 18 ml agar | 0.25 | 0.250 |
| | - 1.0 ml into 18 ml agar | 0.125 | 0.132 |
| | - 0.5 ml into 18 ml agar | 0.062 | 0.068 |
| Solution E 0.31 µg/ml | - 2.0 ml into 18 ml agar | 0.031 | 0.031 |
| | - 1.0 ml into 18 ml agar | 0.016 | 0.016 |
| | - 0.5 ml into 18 ml agar | 0.008 | 0.008 |

NOTE: The "nominal" test article concentrations shown above were achieved to within 10% of the stated value. This is an acceptable degree of accuracy in a doubling dilution series.

4.7.5 Immediately after test article addition, each aliquot was gently mixed by inversion: bubble formation was avoided. Each aliquot was then poured promptly into a single sterile petri dish, avoiding cooling and solidification of agar in the bottle. Each petri dish was labelled with the study number and test article concentration.

4.7.6 Three control plates (containing no test article) were also prepared by adding 2.0 ml of deionized water in place of test article dilution. An additional volume of "control" agar was prepared and allowed to solidify in its bottle.

4.7.7 All plates were allowed to cool until the agar had solidified. Prepared plates were used on the day of preparation.

4.7.8 The pH of each agar batch was checked after autoclaving, addition of blood and cooling to room temperature. This was achieved by immersing the electrode of a calibrated pH meter in the reserved 20 ml volume of "control" agar, after allowing it to solidify in its bottle. All agar used in the study conformed to the specification of pH 7.0 ±0.1.

4.8 <u>Procedure: preparation of bacterial inocula</u>

4.8.1 Each inoculated culture medium was labelled with the study number, organism code number and date of subculture on the bottle or petri dish.

4.8.2 Each test strain and control strain was subcultured from frozen stocks on to FAA. All plate cultures were incubated at 35±1°C until discrete bacterial colonies were readily visible on the agar. Typical incubation times were 24 to 48 hours for *Fusobacterium* strains, and 3 to 5 days for *Prevotella* and *Porphyromonas* strains.

4.8.3 Each incubated plate was carefully inspected to confirm the presence of a pure culture, and further subcultures were prepared if necessary.

4.8.4 From each incubated culture, three to five colonies of typical morphology was selected and sampled by touching with a sterile bacteriological loop. Bacterial cells collected in this way was suspended in a sterile, pre-reduced aliquot of MRD, with vortex mixing, until homogenous turbidity equivalent to that of a 0.5 McFarland Standard was attained. Comparison with the McFarland turbidity standard was made against a white card bearing contrasting black lines. A bacterial suspension adjusted in this way contained approximately $1 \times 10^8$ cfu per ml.

4.8.5 All standardized suspensions were used within 30 minutes of preparation.

4.9 Procedure: inoculation of MIC plates

4.9.1 Each set of prepared agar plates was inoculated with standardized bacterial suspensions using an automatic multipoint inoculator fitted with a suitable inoculating head and tray. Up to 25 bacterial strains (including controls) were tested on one series of MIC plates.

4.9.2 Each culture (including the *B. fragilis* and *B. thetaiotaomicron* controls) were assigned a position in the tray and these positions were recorded diagrammatically to ensure correct plate reading.

4.9.3 The tray was properly fitted on the multipoint inoculator stage. Sterile inoculating pins and one marker pin was fitted in the inoculator head. Test plates were inoculated with approximately 2 µl of each culture using the inoculating pins. The growth control plate (no antimicrobial agent) was inoculated first and then, starting with the lowest concentration, plates containing test compound were inoculated. A second growth control plate was inoculated last to confirm the absence of contamination or significant carry over of test compound during inoculation. An uninoculated plate was used as a sterile agar control.

4.10 Procedure: incubation of test plates

Inoculated plates were left undisturbed until the inoculum spots had been absorbed into the agar. Plates was then inverted and incubated anaerobically (*i.e.* remaining within the anaerobic workstation) at 35±1°C for 42 to 48 h in the first instance.

4.11 Procedure: plate reading and determination of MIC

4.11.1 MIC plates were read against a dark non-reflecting surface. The MIC of each test article was recorded as the lowest concentration that completely inhibited growth, disregarding a single colony or a faint haze caused by the inoculum. Raw data in the form of growth/no growth (+/-) of each inoculum at each antibiotic concentration was recorded in the laboratory notebook in tabular form.

4.11.3 In the case of *Prevotella* and *Porphyromonas* strains, it was not possible to determine MIC results after 42 – 48 h incubation. MIC plates were re-incubated and inspected daily until sufficient growth had occurred to permit interpretation. The total incubation period was approximately 4 days.

4.11.4 Test system performance was monitored on the basis of MIC results obtained for the two *Bacteroides* control strains, which was included in each experiment. For each test article, MIC results obtained against a given control strain should fall within ±1 doubling dilution either side of a central value.

5. RESULTS AND DISCUSSION

5.1 Minimum Inhibitory Concentrations (MICs) of pradofloxacin and des-cyano-pradofloxacin against each bacterial strain used in the present study are presented in Tables 1 to 3 (one table for each bacterial group).

5.2 For each bacterial group and for the entire panel of 31 strains, antibacterial activity is summarized in Tables 4 to 5 (one table for each test article) on the basis of MIC range, $MIC_{50}$, $MIC_{90}$ and geometric mean MIC. These parameters are calculated as follows:

- For any group of bacterial strains, $MIC_{50}$ is the minimum concentration of test article at which growth of 50% of strains is inhibited. For a group of $n$ MIC results ranked from lowest to highest, $MIC_{50}$ is chosen as the $i^{th}$ value, where $i/n \geq 50\%$ and $(i-1)/n < 50\%$.

- For any group of bacterial strains, $MIC_{90}$ is the minimum concentration of test article at which growth of 90% of strains is inhibited. For a group of $n$ MIC results ranked from lowest to highest, $MIC_{90}$ is chosen as the $j^{th}$ value, where $j/n \geq 90\%$ and $(j-1)/n < 90\%$. $MIC_{90}$ can only be calculated where $n \geq 10$.

- Geometric mean = antilog $\left(\frac{\Sigma \log y}{n}\right)$

  where $n$ is the number of bacterial strains in the group and $y_1 \ldots y_n$ are the individual MIC values for each of the strains 1 to $n$

5.3 Both test articles exerted measurable antibacterial activity against all strains tested in the present study. On the basis of the summary MIC results presented in Table 4 and 5, both of the pradofloxacin derivatives exerted greatest antibacterial activity against *Porphyromonas* strains, and lest activity against *Fusobacterium* strains.

5.4 Pradofloxacin was demonstrated to be more active than des-cyano-pradofloxacin against the bacterial strains tested in this study. Based on the geometric mean MIC, pradofloxacin activity was six-fold higher than that of the des-cyano derivative.

5.5 The MIC of each test article against the control strains is recorded in Table 6 below. For each test article, the MIC against a given *Bacteroides* control strain was reproducible within ±1 doubling dilution. Furthermore, control strain MICs for pradofloxacin fell within the proposed QC limits published in CLSI document M31-A3, as follows:

*B. fragilis* ATCC 25285 – proposed QC range 0.06 to 0.25 µg/ml
*B. thetaiotaomicron* ATCC 29741 – proposed QC range 0.5 to 2 µg/ml

## 6. CONCLUSIONS

6.1 The activity of each test article was greater against *Porphyromonas* isolates than against *Prevotella* or *Fusobacterium* isolates.

6.3 Pradofloxacin was demonstrated to be more active than des-cyano-pradofloxacin against anaerobic bacteria implicated in periodontal disease. Based on the geometric mean MIC, pradofloxacin activity was six-fold higher than that of the des-cyano derivative. Thus, it can be concluded that the high in-vitro activity of pradofloxacin against anaerobic bacteria is attributable to the cyano group at the C-8 position of this new veterinary fluoroquinolone.

## 7. TABULATED DATA

Table 1 Minimum Inhibitory Concentration (MIC) of pradofloxacin and des-cyano-pradofloxacin against *Fusobacterium* isolates

| DWC code | Description | Pradofloxacin MIC (µg/ml) | Des-cyano-pradofloxacin MIC (µg/ml) |
|---|---|---|---|
| 5736 | *Fusobacterium nucleatum* | 0.25 | 0.5 |
| 5763 | *Fusobacterium nucleatum* | 0.125 | 0.5 |
| 5780 | *Fusobacterium nucleatum* | 0.062 | 0.5 |
| 5783 | *Fusobacterium nucleatum* | 0.125 | 1 |
| 5785 | *Fusobacterium nucleatum* | 0.062 | 0.5 |
| 5790 | *Fusobacterium nucleatum* | 0.062 | 0.5 |
| 5792 | *Fusobacterium nucleatum* | 0.062 | 0.5 |
| 6924 | *Fusobacterium varium* | 0.5 | 4 |
| 7592 | *Fusobacterium ulcerans* | 0.5 | 4 |
| 5732 | *Fusobacterium necrogenes* | 0.5 | 4 |
| 6957 | *Fusobacterium naviforme* | 0.5 | 4 |

Table 2 Minimum Inhibitory Concentration (MIC) of pradofloxacin and des-cyano-pradofloxacin against *Prevotella* isolates

| DWC code | Description | Pradofloxacin MIC (µg/ml) | Des-cyano-pradofloxacin MIC (µg/ml) |
|---|---|---|---|
| 10397 | *Prevotella denticola* | 0.062 | 0.5 |
| 10504 | *Prevotella denticola* | 0.125 | 0.5 |
| 10891 | *Prevotella intermedia* | 0.062 | 0.5 |
| 10761 | *Prevotella denticola* | 0.125 | 1 |
| 11027 | *Prevotella intermedia* | 0.062 | 0.5 |
| 11074 | *Prevotella intermedia* | 0.016 | 0.062 |
| 11089 | *Prevotella intermedia* | 0.062 | 0.5 |
| 11487 | *Prevotella denticola* | 0.062 | 0.25 |
| 12346 | *Prevotella intermedia* | 0.062 | 0.25 |
| 16229 | *Prevotella intermedia* | 0.062 | 0.5 |

Table 3 Minimum Inhibitory Concentration (MIC) of pradofloxacin and des-cyano-pradofloxacin against *Porphyromonas* isolates

| DWC code | Description | Pradofloxacin MIC (µg/ml) | Des-cyano-pradofloxacin MIC (µg/ml) |
|---|---|---|---|
| 10394 | *Porphyromonas gingivalis* | 0.062 | 0.5 |
| 10686 | *Porphyromonas gingivalis* | 0.031 | 0.25 |
| 10791 | *Porphyromonas gingivalis* | 0.062 | 0.25 |
| 10799 | *Porphyromonas gingivalis* | 0.062 | 0.5 |
| 10805 | *Porphyromonas gingivalis* | 0.062 | 0.5 |
| 11062 | *Porphyromonas gingivalis* | 0.031 | 0.031 |
| 11040 | *Porphyromonas gingivalis* | 0.062 | 0.5 |
| 11834 | *Porphyromonas gingivalis* | 0.062 | 0.5 |
| 11837 | *Porphyromonas gingivalis* | 0.062 | 0.5 |
| 12313 | *Porphyromonas gingivalis* | 0.062 | 0.25 |

Table 4 Summary of pradofloxacin antibacterial activity against anaerobic bacteria from cases of canine periodontal disease

| Bacterial species (number of strains) | Summary MIC parameters: pradofloxacin (µg/ml) | | | |
|---|---|---|---|---|
| | MIC range | $MIC_{50}$ | $MIC_{90}$ | Geometric mean MIC |
| *Fusobacterium* spp. (11) | 0.062 to 0.5 | 0.125 | 0.5 | 0.171 |
| *Prevotella* spp. (10) | 0.016 to 0.125 | 0.062 | 0.125 | 0.0623 |
| *Porphyromonas* spp. (10) | 0.031 to 0.062 | 0.062 | 0.062 | 0.0540 |
| All strains (31) | 0.016 to 0.5 | 0.062 | 0.5 | 0.0851 |

Table 5 Summary of des-cyano-pradofloxacin antibacterial activity against anaerobic bacteria from cases of canine periodontal disease

| Bacterial species (number of strains) | Summary MIC parameters: des-cyano-pradofloxacin (µg/ml) | | | |
|---|---|---|---|---|
| | MIC range | $MIC_{50}$ | $MIC_{90}$ | Geometric mean MIC |
| *Fusobacterium* spp. (11) | 0.5 to 4 | 0.5 | 4 | 1.13 |
| *Prevotella* spp. (10) | 0.062 to 1 | 0.5 | 0.5 | 0.379 |
| *Porphyromonas* spp. (10) | 0.031 to 0.5 | 0.5 | 0.5 | 0.308 |
| All strains (31) | 0.031 to 4 | 0.5 | 4 | 0.523 |

Table 6 Minimum Inhibitory Concentration (MIC) pradofloxacin and des-cyano-pradofloxacin against *Bacteroides* control strains.

| DWC code | Strain Description | Test date | MIC Result (µg/ml) | |
|---|---|---|---|---|
| | | | Pradofloxacin | Des-cyano-pradofloxacin |
| DWC 9674 | *Bacteroides fragilis* ATCC 25285 | 25/09/2008 | 0.125 | 1 |
| | | 29/09/2008 | 0.125 | 1 |
| | | 09/10/2008 | 0.25 | 1 |
| | | 14/10/2008 | 0.125 | 1 |
| DWC 14809 | *Bacteroides thetaiotaomicron* ATCC 29741 | 25/09/2008 | 1 | 8 |
| | | 29/09/2008 | 1 | 8 |
| | | 09/10/2008 | 1 | 8 |
| | | 14/10/2008 | 1 | 8 |